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Abstract

HAI-1 was initially identified as cognate inhibitor of matriptase, a membrane-bound serine protease. Paradoxically HAI-1 is also required for matriptase activation, a process that requires sphingosine 1-phosphate (S1P)-mediated translocation of the protease to cell-cell junctions in human mammary epithelial cells. In the current report, we further explored how HAI-1 regulates this protease. First we observed that following S1P treatment HAI-1 was co-translocated with matriptase to cell-cell junctions and that the cellular ratio of HAI-1 to matriptase was maintained during this process. However, when this ratio was changed by cell treatment with HAI-1 siRNA or anti-HAI-1 mAb M19, spontaneous activation of matriptase occurred in the absence of S1P-induced translocation; S1P-induced matriptase activation was also enhanced. These results support a role for HAI-1 in protection of cell from uncontrolled matriptase activation. We next expressed matriptase, either alone or with HAI-1 in breast cancer cells that do not endogenously express either protein. A defect in matriptase trafficking to the cell surface occurred if wild type matriptase was expressed in the absence of HAI-1; this defect appeared to result from matriptase toxicity to cells. Coexpression with matriptase of wild type HAI-1, but not HAI-1 mutants altered in its Kunitz domain 1, corrected the trafficking defect. In contrast, catalytically defective matriptase mutants were normal in their trafficking in the absence of HAI-1. These results are also consistent with a role for HAI-1 to prevent inappropriate matriptase proteolytic activity during its protein synthesis and trafficking. Taken together, these results support multiple roles for HAI-1 to regulate matriptase, including its proper expression, intracellular trafficking, activation, and inhibition.

Table of Contents

Introduction:

The death of women with breast carcinoma mainly results from metastasis. Metastatic breast cancer cells must escape from a primary tumor and migrate through anatomical barriers in order to gain access to the blood or lymphatic system and establish at a new site in the body. Cellular motility and degradation of extracellular matrix (ECM) are two of the major events in breast cancer metastasis and can be promoted by stromal-derived, ECM-degrading protease systems, such as the urokinase type plasminogen activator (uPA) system and by motility factors, such as hepatocyte growth factor (HGF)/scatter factor (SF). In order to understand how breast cancer cells regulate both stromal-derived, ECM degradation and cellular motility for metastasis, we have discovered and characterized in human breast cancer a new epithelial-derived, type 2 integral membrane, serine protease, matriptase, and its cognate inhibitor, a Kunitz-type serine protease (KSPI), a type 1 integral membrane protein which was initially identified as an inhibitor of hepatocyte growth factor activator and named as HAI-1 (1-3). Both matriptase and HAI-1 have been implicated in the regulation of ECM-degradation of cellular motility (4). In the current research plan, we proposed to study the anti-tumor and anti-protease activity of this membrane-bound Kunitz inhibitor.

Body:

During the August 04-July 05 period we had addressed Aim 3 of the original proposal:
Year 4: In the final year, we will continue the characterization of both KSPI-complexes.

In the previous year, we have finished the role of KSPI (now termed HAI-1) in matriptase activation and inhibition. The transfection of HAI-1 in breast cancer cells have been completed and reported in the annual report of first year, and the new HAI-1 complex turns out to be HGF activator complexed with HAI-1. In the current report, we have further investigated how HAI-1 regulated matriptase expression, trafficking and activation in cell biology aspects. These works have been published in American Journal Physiology-Cell Physiology (289:C462-70, 2005). We report here with these works. Please refer to the attached reprint for the Figures

Proportional increase in HAI-1 and matriptase in individual cells and at cell-cell junctions--The close relationship between matriptase and its cognate inhibitor HAI-1 was initially recognized *in vitro* by their concordant expression among cell lines (5), co-incidence on cell surfaces (6) and formation of stable complexes (1;3). We further showed that HAI-1 is required for matriptase activation (7), and that both proteins translocate to activation foci during the activation of the protease (8;9). In 184 A1N4 immortal mammary epithelial cells, while expression levels of matriptase and HAI-1 were not uniform among individual cells, proportional increases or decreases in matriptase and HAI-1 were observed, comparing individual cells (Fig. 1). This relatively constant ratio between matriptase and HAI-1 was even observed when both proteins were translocated to cell-cell junctions during S1P-induced activation of matriptase (Fig. 1). Matriptase tended to accumulate more at the merging points of cell-cell contacts of three or four cells (focal points) (Fig. 1). HAI-1 shared this uneven distribution at cell-cell contacts, with higher levels of matriptase accompanied by higher levels of HAI-1 (Fig. 1). Because matriptase and HAI-1 localization is restricted to common points of cell-cell contact, we sought to further explore the role of HAI-1 in matriptase activation and intracellular trafficking.

Reduced expression of HAI-1 by HAI-1 siRNA caused spontaneous activation of matriptase— We reduced the levels of HAI-1 by HAI-1 siRNA to investigate its impact on matriptase activation. After 48 hr treatment with HAI-1 siRNA, 184 A1N4 expressed much less HAI-1, as examined by immunoblot using anti-HAI-1 mAb M19 (Fig. 2, M19, comparing lane 4 with lane 2). The levels of total matriptase were not altered by HAI-1 siRNA (Fig. 2, M32, comparing lane 4 with lane 2). As expected from previous studies (10;11), activation of matriptase did not occur in these cells in the absence of an exogenous inducer, such as S1P (Fig. 2, M69 lane 2). However, reduced expression of HAI-1 by siRNA in these cells caused spontaneous activation of matriptase (Fig. 2, M69, comparing lane 4 with lane 2). As described previously (9), the activated matriptase was detected in 120- and 85-kDa complexes using mAb M69, which specifically recognizes the activated, two-

chain matriptase. The ratio between 120- and 85-kDa complexes varied from experiment to experiment. As examined by immunofluorescence staining, activated matriptase was detected as a diffuse pattern, with some aggregation (Fig. 3). Because individual cells express different amount of HAI-1 (Fig. 1), the reduction of HAI-1 expression by HAI-1 siRNA, as seen in immunoblot (Fig. 2), can not clearly be demonstrated by immunofluorescence staining (Fig. 3). These data suggest that maintenance of a proper ratio between matriptase and HAI-1 may be essential for prevention of spontaneous activation of matriptase in mammary epithelial cells.

Reduced HAI-1 expression significantly enhanced S1P-induced matriptase activation-- When HAI-1 siRNA-treated cells were induced by S1P for matriptase activation, a significant increase in activated matriptase was observed, compared to that in the control cells (Fig. 2, M69, comparing lane 3 to lane 1). Immunofluorescent staining showed that, after S1P addition to cells, matriptase activation occurred primarily at the junction of multiple cells, and subsequently spread to cell to cell junctions between two adjacent cells. In the HAI-1 siRNA-treated cells, more activated matriptase was detected in an elongated pattern along the cell-cell junctions, compared to in the control cells (Fig. 4). Because the expression levels of both matriptase and HAI-1 varied substantially in individual 184 A1N4 cells, it was difficult to discern the reduced expression of HAI-1-1 by siRNA in some cells. For most S1P-treated cells, HAI-1 was detected as a bright band along the cell-cell junctions, with diffuse areas of staining adjacent to the cell-cell junctions. HAI-1 siRNA caused the disappearance of the areas of diffuse staining in some cells, where the activated matriptase was detected in more elongated patterns. These data suggest that reduced HAI-1 levels result in an increase in the activation of matriptase adjacent to cell-cell junctions, leading to higher total matriptase activation.

Pretreatment of 184 A1N4 cells with the anti-HAI-1 mAb M19 enhances S1P-induced matriptase activation — We further tested if the anti-HAI-1 mAb M19 could affect S1P-induced matriptase activation (Fig. 5). Previously, we showed that pretreatment with anti-matriptase mAb M32, which recognizes the third LDL receptor domain of matriptase, inhibited S1P-induced matriptase activation in 184 A1N4 cells (8). Thus, we incorporated mouse IgG and mAb M32 as the controls. After growth for two days, the cells were preincubated with 5 μg/ml of mouse IgG, M32, or M19, respectively, for 1 hr. These pretreatments of cells with antibodies alone did not cause matriptase activation, as examined by immunoblot, but activated matriptase was observed by immunofluorescence staining in few scattered M19-pretreated cells (data not shown). After stimulating these antibody-pretreated cells with S1P for 30 min, activated matriptase was detected in mouse IgG-pretreated cells, but not in M32-pretreated ones, as examined by immunoblot (Fig. 5A) or by immunofluorescence staining (Fig. 5 B). Much higher levels of activated matriptase were detected in mAb M19-pretreated cells, compared to those in mouse IgG-pretreated ones.

Immunofluorescence staining further revealed that the increase in matriptase activation by mAb M19 resulted from two events: activation occurred in more cells and in a more elongated manner along cell-cell junctions (Fig. 5B). Activated matriptase was detected in almost every single M19-pretreated cell, but only in approximately one half of mouse IgG-pretreated cells. Activated matriptase was detected predominately at the junction of multiple cells in the IgG-pretreated cells, whereas activated matriptase was visualized along nearly the entire cell borders of M19-pretreated cells (Fig. 5B).

HAI-1 is required for expression of matriptase-- Given these complex and well-coordinated relationships between matriptase and HAI-1, it seems likely that HAI-1 may also play a role during matriptase biosynthesis and degradation. This notion was first supported when we attempted to exogenously express matriptase in BT549 breast cancer cells that do not endogenously express either matriptase or HAI-1. When BT549 cells were transiently transfected with wild-type matriptase alone, only a very low level of matriptase protein was detected by western blotting, using anti-matriptase mAb M32 upon long exposure of blots. However, transient transfection with a cDNA coding for HAI-1 resulted in strong expression of exogenous HAI-1 in these cells (Fig. 6). When the protease was co-transfected with the inhibitor, much higher levels of matriptase were seen. The poor expression of matriptase, when the protease was transfected alone, may result from its proteolytic activity, since exogenous expression of matriptase mutants altered in the catalytic triad

(S805A matriptase) or in the substrate binding pocket (D799A matriptase), were achieved in BT549 breast cancer cells, independently of the presence of HAI-1 (Fig. 6). While high levels of matriptase expression were achieved for the matriptase mutant altered in its active site triad, coexpression of the inactive catalytic triad matriptase mutant with wild-type matriptase caused poor expression of both matriptase species (Fig. 6). These results suggest that unopposed matriptase proteolytic activity could be toxic for its expression, and that the presence of HAI-1 corrects this toxic effect, leading to higher levels of matriptase expression.

Immunofluoresence staining using Texas red conjugated phalloidin (actin), together with the anti-matriptase mAb M32 (Fig. 7A), suggested that the poor expression was due to a defect in intracellular trafficking of the protease. Indeed, immunofluorescence using an antibody that recognizes GM130, a Gogi-specific marker, together with Alexa-Fluor®594-conjugated M32 matriptase-specific antibody (Fig. 7D), revealed that matriptase accumulated in a cellular location consistent with the endoplasmic reticulum and Golgi apparatus. This defect in trafficking was corrected by cotransfection with HAI-1, as shown by immunofluorescence (Fig. 7B and E), and resulted in much improved expression of the protease, as shown by western blotting (Fig. 7A). In addition, catalytically inactive mutants of matriptase altered in the catalytic triad (S805A matriptase) were able to traffic in the absence of HAI-1 (Fig 7C).

The proper trafficking and enhanced expression of the matriptase protein when co-transfected with HAI-1 was not a phenomena limited to BT549 cells. Indeed, it was also seen when matriptase and HAI-1 were transfected into MDA MB-231 and MDA MB-435 cancer cells (data not shown). These two cancer cell lines also neither express matriptase nor HAI-1 (5).

The first Kunitz domain, but not the second, is required for HAI-1 to facilitate matriptase intracellular trafficking-- Since the intracellular trafficking of matriptase depended upon HAI-1, we were able to test which domains in HAI-1 were essential for protease expression and trafficking. HAI-1 contains two Kunitz-type serine protease inhibitory domains, Kunitz domain I (at the amino terminus) and Kunitz domain II (at the carboxyl terminus), with an intervening LDL receptor class A domain (12) (Fig. 7).

The Kunitz domain is an approximately 60 amino acid long serine protease inhibitory domain for which the bovine basic pancreatic trypsin inhibitor (BPTI) represents the prototypic structure (13). The P1 residue of Kunitz-type inhibitory domains (the amino acid residue C-terminal to the second conserved cysteine residue) is recognized as the active center, responsible for the inhibitory specificity. For example, the corresponding amino acid residues in the Kunitz domains of HAI-1 are Arg-260 in domain I and Lys-385 in domain II, and therefore these Kunitz domains are predicted to be specific for trypsin-like serine proteases such as matriptase (interacting with Asp-799 in the substrate binding pocket of matriptase). Mutation of Arg-260 will completely abolish the inhibitory activity of a Kunitz domain. Previously, molecular modeling strongly suggested that the first, but not the second, Kunitz domain of HAI-1 is responsible for the inhibition of matriptase (14). In addition to Arg-260, Arg-258 was also suggested to be crucial for the activity of Kunitz domain I. Therefore, we constructed point mutations at these three critical basic residues in Kunitz domains I and II (Arg-258, Arg-260, and Lys-385).

Mutation of critical arginine residues in the first Kunitz domain of HAI-1 (R258L and R260L HAI-1) completely abolished the ability of HAI-1 to facilitate the intracellular trafficking of matriptase (Fig. 8). In co-transfection experiments with these HAI-1 mutants, matriptase was poorly expressed, as determined by M32 western blotting, and did not traffic properly as determined by matriptase immunofluorescence (data not shown). Mutation of a critical basic residue in the second Kunitz domain of HAI-1 (K385L HAI-1), however, did not affect the ability of HAI-1 to facilitate matriptase trafficking, since matriptase expressed well (Fig. 8) and underwent proper intracellular trafficking, as confirmed by matriptase immunofluorescence (data not shown). It should be noted that the R258L and R260L HAI-1 mutants did not express well when transfected with matriptase, in contrast to the K385L HAI-1 mutant (Fig. 8). However, this was due to the defect in intracellular trafficking created by unopposed matriptase activity, and not by poor transfection efficiency, as

determined by immunofluorescence staining (data not shown). These results suggest that matriptase proteolytic activity, which could result from spontaneous activation in the absence of HAI-1, generally interferes with the trafficking of transmembrane proteins when transfected in the absence of HAI-1.

Key research accomplishments:

- Proportional increase in HAI-1 and matriptase in individual cells and at cell-cell junctions
- Reduced expression of HAI-1 by HAI-1 siRNA caused spontaneous activation of matriptase
- Reduced HAI-1 expression significantly enhanced S1P-induced matriptase activation
- Pretreatment of 184 A1N4 cells with the anti-HAI-1 mAb M19 enhances S1P-induced matriptase activation.
- HAI-1 is required for expression of matriptase.
- The first Kunitz domain, but not the second, is required for HAI-1 to facilitate matriptase intracellular trafficking

Reportable outcomes:

1. Oberst, M.D., Anders, J., Benaud, C., Lin, C.-Y., Dickson, R.D., and Johnson, M.D., (2001) Over-expression of the hepatocyte growth factor inhibitor (HAI-1) in human breast cancer cells: Effects on cell growth, morphology, and motility. 2nd General Meeting of International Protease Society, Muenchen, German.
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6. Lee M.-S., Kiyomiya K., Benaud C., Dickson R.B., and Lin C.-Y. (2005) Simultaneous activation and HAI-1-mediated inhibition of matriptase induced at activation foci in human mammary epithelial cells. *Am J Physiol Cell Physiol.* 288:C932-C941.
7. Oberst MD, Chen LY, Kiyomiya KI, Williams CA, Lee MS, Johnson MD, Dickson RB, Lin CY. (2005) HAI-1 regulates activation and expression of matriptase, a membrane-bound serine protease. *Am J Physiol Cell Physiol.* 289:C462-470.

Conclusion:

In the current report, we further explored the role of HAI-1 in the regulation of matriptase activation by reducing the expression levels of HAI-1 or sequestering HAI-1 using an anti-HAI-1 monoclonal antibody. Interestingly, reduced HAI-1 expression or availability caused spontaneous activation of matriptase and enhanced activation of matriptase by S1P. Furthermore, this spontaneous activation of matriptase was closely

associated with a defect in the trafficking of the protease, and coexpression of HAI-1 with matriptase corrected this defect. These results suggest that regulation of matriptase by HAI-1 may occur at multiple levels, including protein biosynthesis, intracellular trafficking, prevention of uncontrolled spontaneous activation, regulated activation, and ectodomain shedding.

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Appendices: A reprint of the following paper is provided:
Oberst MD, Chen LY, Kiyomiya KI, Williams CA, Lee MS, Johnson MD, Dickson RB, Lin CY. (2005) HAI-1 regulates activation and expression of matriptase, a membrane-bound serine protease. *Am J Physiol Cell Physiol.* 289:C462-470

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HAI-1 regulates activation and expression of matriptase, a membrane-bound serine protease

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Oberst, Michael D., Li-Yuan L. Chen, Ken-Ichi Kiyomiya, Cicely A. Williams, Ming-Shyue Lee, Michael D. Johnson, Robert B. Dickson, and Chen-Yong Lin. HAI-1 regulates activation and expression of matriptase, a membrane-bound serine protease. *Am J Physiol Cell Physiol* 289: C462–C470, 2005. First published March 30, 2005; doi:10.1152/ajpcell.00076.2005.—Hepatocyte growth factor activator inhibitor-1 (HAI-1) was initially identified as cognate inhibitor of matriptase, a membrane-bound serine protease. Paradoxically, HAI-1 is also required for matriptase activation, a process that requires sphingosine 1-phosphate (S1P)-mediated translocation of the protease to cell-cell junctions in human mammary epithelial cells. In the present study, we further explored how HAI-1 regulates this protease. First, we observed that after S1P treatment HAI-1 was cotranslocated with matriptase to cell-cell junctions and that the cellular ratio of HAI-1 to matriptase was maintained during this process. However, when this ratio was changed by cell treatment with HAI-1 small interfering RNA or anti-HAI-1 MAb M19, spontaneous activation of matriptase occurred in the absence of S1P-induced translocation; S1P-induced matriptase activation was also enhanced. These results support a role for HAI-1 in protection of cell from uncontrolled matriptase activation. We next expressed matriptase, either alone or with HAI-1 in breast cancer cells that do not endogenously express either protein. A defect in matriptase trafficking to the cell surface occurred if wild-type matriptase was expressed in the absence of HAI-1; this defect appeared to result from matriptase toxicity to cells. Coexpression with matriptase of wild-type HAI-1, but not HAI-1 mutants altered in its Kunitz domain 1, corrected the trafficking defect. In contrast, catalytically defective matriptase mutants were normal in their trafficking in the absence of HAI-1. These results are also consistent with a role for HAI-1 to prevent inappropriate matriptase proteolytic activity during its protein synthesis and trafficking. Taken together, these results support multiple roles for HAI-1 to regulate matriptase, including its proper expression, intracellular trafficking, activation, and inhibition.

protease-activated receptor-2; hepatocyte growth factor; urokinase; sphingosine 1-phosphate; Kunitz domain

HEPATOCYTE GROWTH FACTOR (HGF) activator inhibitor 1 (HAI-1) is a type 1 integral membrane, Kunitz-type serine protease inhibitor (25). HAI-1 contains two Kunitz domains and a low-density lipoprotein (LDL) receptor class A domain. HAI-1 was initially identified as an inhibitor of HGF activator (25), a liver-derived, blood-borne serine protease, which is a potent activator of pro-HGF (20). Subsequently, HAI-1 was purified from human milk as a complex with matriptase, a multidomain, transmembrane serine protease of the S1 trypsin-like family (11, 15, 16, 29, 30). Matriptase has been implicated in cancer invasion and metastasis by serving as a cell surface activator for urokinase-type plasminogen activator, HGF, and protease-activated receptor 2 (12, 14, 26, 28). A matriptase-specific inhibitory drug has recently been shown to suppress the growth of androgen-independent human prostate cancer cells as tumors in a xenograft model (7). Furthermore, matriptase was shown to be essential for the epidermal cell maturation of profilaggrin, a large (>300 kDa) insoluble phosphoprotein involved in terminal differentiation of keratinocytes (19). The protease is also required for postnatal survival, epidermal barrier function, hair follicle development, and thymic homeostasis in mice (18). Matriptase belongs to the type II transmembrane serine protease group. Members of the serine protease group are characterized by an amino-terminal transmembrane domain and multiple extracellular domains, in addition to the conserved extracellular serine protease catalytic domain (8, 21, 27). Both matriptase and HAI-1 are expressed in vivo by the epithelial components of most epithelium-containing tissues (10, 23) and coincided on the surfaces of cultured breast cancer cells (3). Given the formation of tightly bound complexes, coexpression by epithelial elements in vivo, and coincidence on the cell surface, matriptase is a physiological target protease of HAI-1.

Whereas HAI-1 functions as the physiological inhibitor of matriptase, this inhibitor is also involved in matriptase activation (24). Like most other serine proteases, activation of matriptase requires cleavage at its canonical activation motif to convert a single-chain zymogen to the two-chain active enzyme (1). However, compared with most other serine proteases, in which activation is conducted by other active proteases, matriptase activation depends on its own active site triad (24). Therefore, matriptase must transactivate itself, whereby interactions between at least two matriptase zymogens, and possibly other proteins, such as HAI-1, leads to the activation cleavage (24). This hypothesis has been further supported by the fact that in immortal human mammary epithelial cells, matriptase and HAI-1 are cotranslocated and accumulated at activation foci, either at cell-cell junctions or vesicle-like structures, during matriptase activation induced, respectively, by blood-borne sphingosine 1-phosphate (S1P) (1, 2, 9) or suramin, a sulfide-rich chemical (13). The involvement of HAI-1 in matriptase activation therefore provides an efficient mechanism to inhibit active matriptase. Consequently, activated matriptase is almost exclusively detected in complexes

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bound to HAI-1 (13). The dual functions of HAI-1 in both matriptase activation and inhibition suggest a critical role of the inhibitor in the regulation of matriptase function, not only in suppression of undesired matriptase proteolysis, but also in the regulation of matriptase activation. In the present study, we further explored the role of HAI-1 in the regulation of matriptase activation by reducing the expression levels of HAI-1 or sequestering HAI-1 using an anti-HAI-1 monoclonal antibody. Interestingly, reduced HAI-1 expression or availability caused spontaneous activation of matriptase and enhanced activation of matriptase by S1P. Furthermore, this spontaneous activation of matriptase was closely associated with a defect in the trafficking of the protease, and coexpression of HAI-1 with matriptase corrected this defect. These results suggest that regulation of matriptase by HAI-1 may occur at multiple levels, including protein biosynthesis, intracellular trafficking, prevention of uncontrolled spontaneous activation, regulated activation, and ectodomain shedding.

MATERIALS AND METHODS

Chemicals and reagents. Formaldehyde solution was purchased from EM Science (EM Industries, Bibbstown, NJ). Cytochalasin D, suramin, and Ro-31–8220 were obtained from Biomol (Plymouth Meeting, PA). Sphingosine 1-phosphate (S1P) was obtained from Avanti Polar Lipids (Alabaster, AL). S1P was prepared at a 10 μg/ml mixture in a solution containing BSA (1 mg/ml), and was used to treat cells at 50 ng/ml. All other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

Cell culture conditions. Immortalized 184 A1N4 human mammary epithelial cells were a gift from Dr. Martha Stampfer (Lawrence Berkeley National Laboratory, Berkeley, CA), and maintained as described in a previous study (1). BT549 human breast cancer cells were cultured in Iscove's minimal essential media (Invitrogen, Rockville, MD) supplemented with 5% fetal bovine serum in a humidified chamber at 37°C and 5% CO_2.

Monoclonal antibodies and Western blot analysis. Human matriptase protein was detected using the M32 monoclonal antibody, that recognizes the third LDL receptor domain of matriptase and can interact with both the latent (one chain) and activated (two chain) forms of the protease (9, 15). Activated matriptase was detected by the M69 monoclonal antibody (MAb), which recognizes an epitope present only in the activated, two-chain form of the enzyme (1, 2). Human HAI-1 was detected with the use of the HAI-1-specific monoclonal antibody M19 (15). The latent form matriptase could be detected by MAb M32 at either 70 kDa (NH_2 terminal processed form) or at 93 kDa (the full-length matriptase, seen mainly with forced expression of matriptase). The activated matriptase was detected by MAbs M69 or M19 at 120 kDa (70-kDa active matriptase plus full-length HAI-1) or at 85 kDa (the full-length HAI-1 complexed with the serine protease domain of matriptase). M32 MAb only recognizes the 120-kDa complex, but not the 85-kDa complex (13). M19 MAb also detects the 55-kDa full-length HAI-1.

Immunofluorescence microscopy. Cells were plated onto microcover glasses and grown for 2 days. Cells received different treatments, as indicated in each figure. Cells were then fixed and permeabilized in phosphate-buffered saline containing in 0.05% Triton X-100 and 3.7% formaldehyde for 20 min at room temperature, followed by three washes with phosphate-buffered saline. Matriptase, activated matriptase, and HAI-1 were detected with Alexa Fluor dye-conjugated M32, M69, and M19 monclonal antibodies, respectively. F-actin was visualized with Texas red-conjugated phalloidin (Molecular Probes), and nuclei were stained with 4,6-diamidino-2-phenylindole. Golgi were visualized using the GM130 monoclonal antibody (BD Biosciences, Palo Alto, CA), followed by a secondary goat anti-mouse FITC-conjugated antibody. For costaining of Golgi and matriptase, the GM130 antibody staining was followed by Alexa Fluor 594-conjugated M32 antibody and a 100-fold excess of mouse IgG to prevent the cross-reaction of any GM130-bound goat anti-mouse FITC with the Alexa Fluor 594-conjugated M32 monoclonal antibody. After fluorescent staining, cover glasses were mounted with Prolong Antifade (Molecular Probes), and the fluorescent images were captured by the MetaVue software package (Molecular Devices) on a Nikon Eclipse E600 digital fluorescence microscope.

HAI-1 small interfering RNA. Two independent small interfering (si)RNA target sites were selected in HAI-1 using standard design criteria (5): HAI-1A, AACUGCAACUUGGCGCUAGU; and HAI-1B, AGAUCUGCAAG AGUUUCGUU. Synthetic siRNA oligos against these targets were purchased from Dharmacon in a duplex-ready 2′-angiotensin-converting enzyme-protected form and prepared and stored according to the manufacturer's instructions. Cells plated on 18-mm-thick glass coverslips, for immunofluorescence microscopy, or in tissue culture dishes, were transfected with the siRNA using oligofectamine and Opti-MEM medium (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Identical results were obtained with HAI-1A and HAI-1B (11), and control experiments that used siRNA directed against an irrelevant gene had no effect.

Constructs and transfections. The cDNA clones for the full-length human matriptase coding sequence, or the full-length human HAI-1 coding sequence in the vector pcDNA3.1 (Invitrogen), were used in transient transfections. These constructs were also used to make site-directed mutants of matriptase or HAI-1. For making site-directed mutations, the QuikChange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used with primers containing appropriate nucleotide changes, according to the manufacturer's protocol. All deletion mutants were confirmed by DNA sequencing. For each mutant, at least two separately isolated expression constructs were isolated and tested in transient transfections. Transient transfection of human matriptase or human HAI-1 constructs (wild type or mutant) was accomplished using Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN), according to the manufacturer's protocol. When conducting cotransfections, the amount of DNA used with the transfection reagent was kept constant for each individual transfection by including empty vector pcDNA3.1 DNA where appropriate.

RESULTS

Proportional increase in HAI-1 and matriptase in individual cells and at cell-cell junctions. The close relationship between matriptase and its cognate inhibitor HAI-1 was initially recognized in vitro by their concordant expression among cell lines (22), coincidence on cell surfaces (3), and formation of stable complexes (15, 17). We further showed that HAI-1 is required for matriptase activation (24), and that both proteins translocate to activation foci during the activation of the protease (9, 13). In 184 A1N4 immortal mammary epithelial cells, while expression levels of matriptase and HAI-1 were not uniform among individual cells, proportional increases or decreases in matriptase and HAI-1 were observed compared with individual cells (Fig. 1). This relatively constant ratio between matriptase and HAI-1 was even observed when both proteins were translocated to cell-cell junctions during S1P-induced activation of matriptase (Fig. 1). Matriptase tended to accumulate more at the merging points of cell-cell contacts of three or four cells

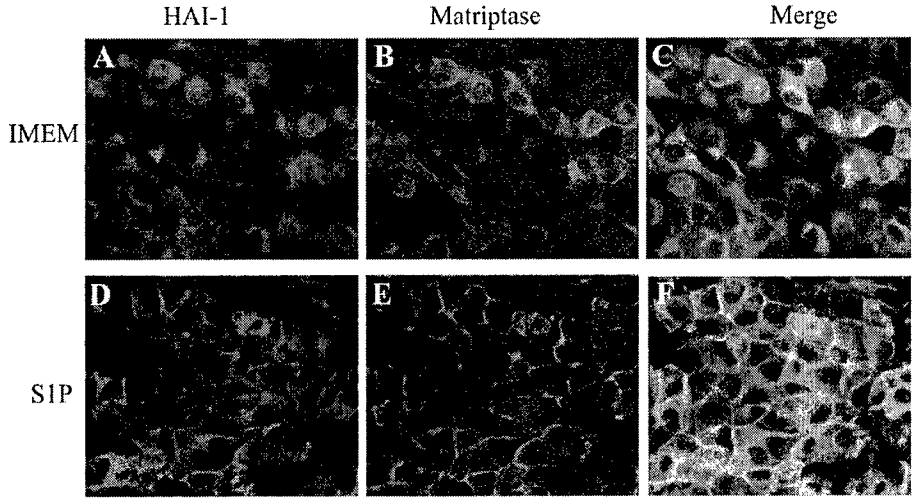

Fig. 1. Proportional increase in hepatocyte growth factor activator inhibitor-1 (HAI-1) and matriptase. Serum-starved 184 A1N4 cells were stimulated with Iscove's minimal essential media (IMEM) (*A–C*) or spingosine 1-phosphate (S1P; 50 ng/ml) for 30 min (*D–F*). Cells were stained for HAI-1 with Alexa Fluor 488-conjugated monoclonal antibody (MAb), M19 (*A* and *D*, green), and for total matriptase with Alexa Fluor 647-conjugated MAb, M32 (*B* and *E*, red). *C* and *F* represent merged images.

(focal points) (Fig. 1). HAI-1 shared this uneven distribution at cell-cell contacts, with higher levels of matriptase accompanied by higher levels of HAI-1 (Fig. 1). Because matriptase and HAI-1 localization is restricted to common points of cell-cell contact, we sought to further explore the role of HAI-1 in matriptase activation and intracellular trafficking.

Reduced expression of HAI-1 by HAI-1 siRNA caused spontaneous activation of matriptase. We reduced the levels of HAI-1 by HAI-1 siRNA to investigate its impact on matriptase activation. After 48 h of treatment with HAI-1 siRNA, 184 A1N4 expressed much less HAI-1, as examined by immunoblot using anti-HAI-1 MAb M19 (Fig. 2; M19, comparing *lane 4* with *lane 2*). The levels of total matriptase were not altered by HAI-1 siRNA (Fig. 2; M32, comparing *lane 4* with *lane 2*). As expected from previous studies (1, 2), activation of matriptase did not occur in these cells in the absence of an exogenous inducer, such as S1P (Fig. 2, M69, *lane 2*). However, reduced expression of HAI-1 by siRNA in these cells caused spontaneous activation of matriptase (Fig. 2; M69, comparing *lane 4* with *lane 2*). As described previously (13), the activated matriptase was detected in 120- and 85-kDa complexes using MAb M69, which specifically recognizes the activated, two-chain matriptase. The ratio between 120- and 85-kDa complexes varied from experiment to experiment. As examined by immunofluorescence staining, activated matriptase was detected as a diffuse pattern with some aggregation (Fig. 3). Because individual cells express different amount of HAI-1 (Fig. 1), the reduction of HAI-1 expression by HAI-1 siRNA, as seen in immunoblot (Fig. 2), cannot clearly be demonstrated by immunofluorescence staining (Fig. 3). These data suggest that maintenance of a proper ratio between matriptase and HAI-1 may be essential for prevention of spontaneous activation of matriptase in mammary epithelial cells.

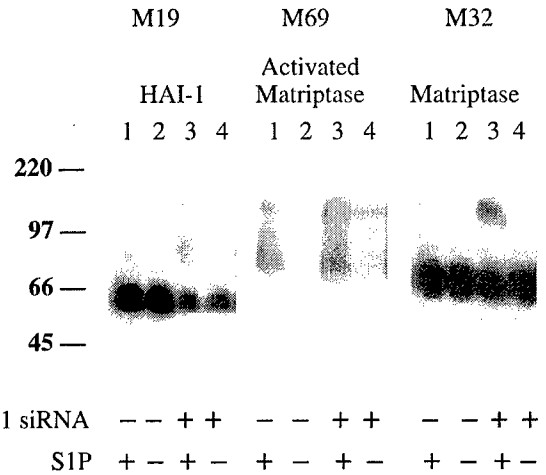

Fig. 2. Transfection of HAI-1 small inhibitory (si)RNA caused spontaneous activation and enhanced S1P-induced activation of matriptase. Serum-starved 184 A1N4 cells were cultured for 24 h, and then transfected with HAI-1 siRNA for 48 h. Control cells were treated with oligofectamin alone. Cells were then incubated with 50 ng/ml S1P for 30 min and equal amounts of total cell lysates were analyzed by Western blot analysis using anti-HAI-1 MAb M19, anti-activated matriptase MAb M69, or anti-matriptase MAb M32.

Reduced HAI-1 expression significantly enhanced S1P-induced matriptase activation. When HAI-1 siRNA-treated cells were induced by S1P for matriptase activation, a significant increase in activated matriptase was observed, compared with that in the control cells (Fig. 2; M69, comparing *lane 3* with *lane 1*). Immunofluorescent staining showed that after S1P addition to cells, matriptase activation occurred primarily at the junction of multiple cells, and subsequently spread to cell-cell junctions between two adjacent cells. In the HAI-1 siRNA-treated cells, more activated matriptase was detected in an elongated pattern along the cell-cell junctions, compared with control cells (Fig. 4). Because the expression levels of both matriptase and HAI-1 varied substantially in individual 184 A1N4 cells, it was difficult to discern the reduced expression of HAI-1–1 by siRNA in some cells. For most S1P-treated cells, HAI-1 was detected as a bright band along the cell-cell junctions, with diffuse areas of staining adjacent to the cell-cell junctions. HAI-1 siRNA caused the disappearance of the areas of diffuse staining in some cells, where the activated matriptase was detected in more elongated patterns. These data suggest that reduced HAI-1 levels result in an increase in the activation of matriptase adjacent to cell-cell junctions, leading to higher total matriptase activation.

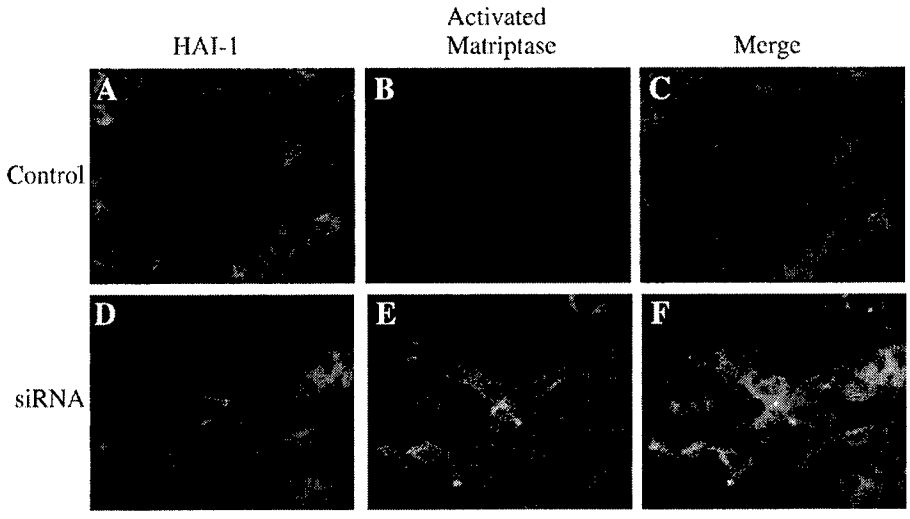

Fig. 3. HAI-1 siRNA caused spontaneous activation of matriptase. Serum-starved 184 A1N4 cells were cultured for 24 h, and then transfected with HAI-1 siRNA for 48 h. Transfection control cells were treated with oligofectamin alone. Cells were stained for HAI-1 with Alexa Fluor 488-conjugated MAb, M19 (*A* and *D*, green), and for activated matriptase with Alexa Fluor 647-conjugated MAb, M69 (*B* and *E*, red). *C* and *F* represent merged images.

Pretreatment of 184 A1N4 cells with anti-HAI-1 MAb M19 enhances S1P-induced matriptase activation. We further tested whether the anti-HAI-1 MAb M19 could affect S1P-induced matriptase activation (Fig. 5). Previously, we showed that pretreatment with anti-matriptase MAb M32, which recognizes the third LDL receptor domain of matriptase, inhibited S1P-induced matriptase activation in 184 A1N4 cells (9). Thus we incorporated mouse IgG and MAb M32 as controls. After being grown for 2 days, the cells were preincubated with 5 μg/ml of mouse IgG, M32, or M19, respectively, for 1 h. These pretreatments of cells with antibodies alone did not cause matriptase activation, as examined by immunoblot, but activated matriptase was observed by immunofluorescence staining in few scattered M19-pretreated cells (data not shown). After these antibody-pretreated cells were stimulated with S1P for 30 min, activated matriptase was detected in mouse IgG-pretreated cells, but not in M32-pretreated ones, as examined by immunoblot (Fig. 5*A*) or by immunofluorescence staining (Fig. 5*B*). Much higher levels of activated matriptase were detected in MAb M19-pretreated cells, compared with those in mouse IgG-pretreated ones.

Immunofluorescence staining further revealed that the increase in matriptase activation by MAb M19 resulted from two events: activation occurred in more cells and in a more elongated manner along cell-cell junctions (Fig. 5*B*). Activated matriptase was detected in almost every single M19-pretreated cell, but only in approximately one-half of mouse IgG-pretreated cells. Activated matriptase was detected predominately at the junction of multiple cells in the IgG-pretreated cells, whereas activated matriptase was visualized along nearly the entire cell borders of M19-pretreated cells (Fig. 5*B*).

HAI-1 is required for expression of matriptase. Given these complex and well-coordinated relationships between matriptase and HAI-1, it seems likely that HAI-1 may also play a role during matriptase biosynthesis and degradation. This notion was first supported when we attempted to exogenously express matriptase in BT549 breast cancer cells that do not endogenously express either matriptase or HAI-1. When BT549 cells were transiently transfected with wild-type matriptase alone, only a very low level of matriptase protein was detected by Western blot analysis

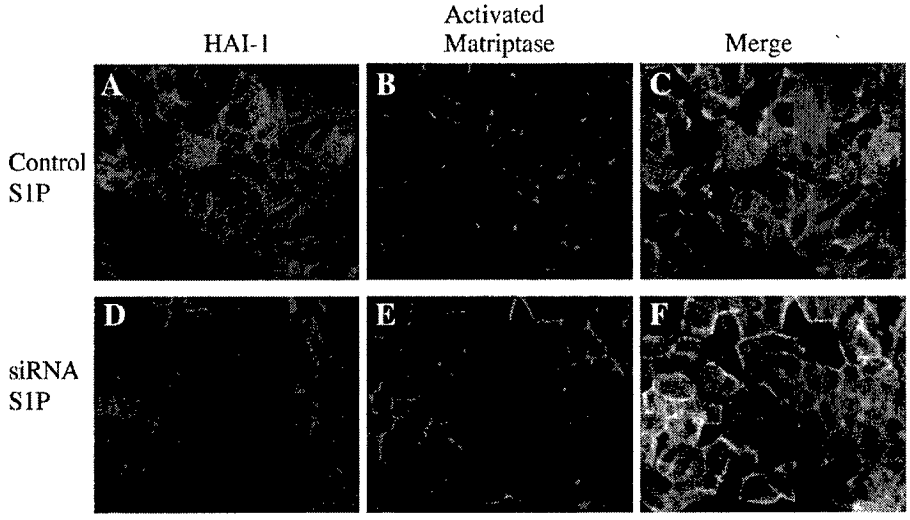

Fig. 4. HAI-1 siRNA enhanced S1P-induced matriptase activation. Serum-starved 184 A1N4 cells were cultured for 24 h, and then transfected with HAI-1 siRNA for 48 h. Transfection control cells were treated with oligofectamin alone. Cells were treated with S1P (50 ng/ml) for 30 min and stained for HAI-1 with Alexa Fluor 488-conjugated MAb, M19 (*A* and *D*; green), and for active from matriptase with Alexa Fluor 647-conjugated MAb, M69 (*B* and *E*; red). *C* and *F* represent merged images.

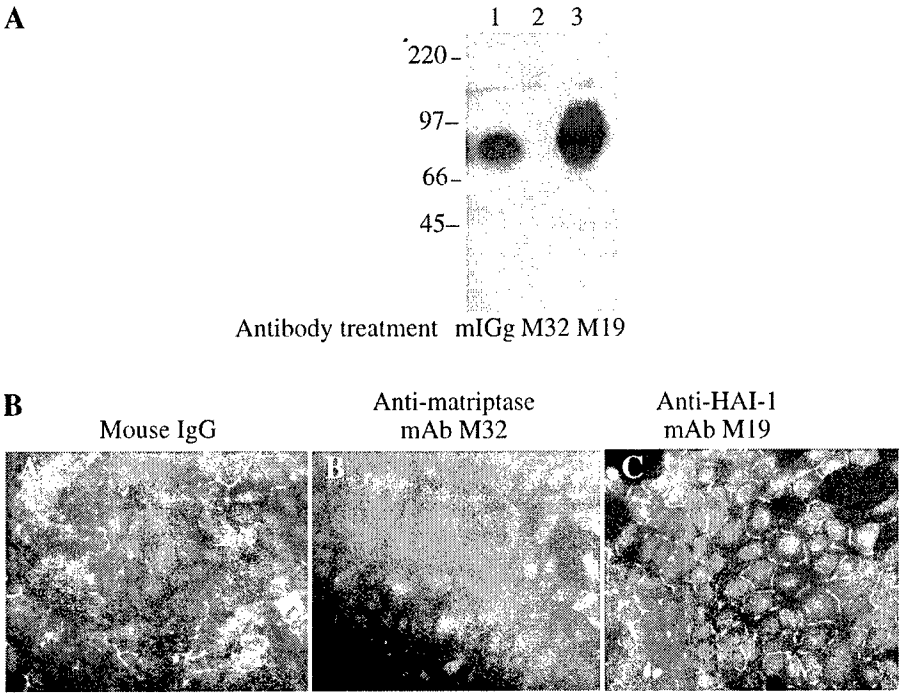

Fig. 5. Anti-HAI-1 antibody, M19, enhanced matriptase activation during S1P induction. Serum-starved 184 A1N4 cells were incubated with either 5 μg/ml of normal mouse IgG, or anti-matriptase MAb, M32, or anti-HAI-1 MAb, M19 for 30 min, and then all stimulated with S1P (50 ng/ml) for 30 min. The levels and distribution of activated matriptase were analyzed by immunoblots with the use of horseradish peroxidase-conjugated MAb M69 (*A*) or by immunofluorescence staining with the use of Alexa Fluor 488-conjugated MAb M69 (*B*).

with the use of anti-matriptase MAb M32 on long exposure of blots. However, transient transfection with a cDNA coding for HAI-1 resulted in strong expression of exogenous HAI-1 in these cells (Fig. 6). When the protease was cotransfected with the inhibitor, much higher levels of matriptase were seen. The poor expression of matriptase, when the protease was transfected alone, may result from its proteolytic activity because exogenous expression of matriptase mutants altered in the catalytic triad (S805A matriptase) or in the substrate binding pocket (D799A

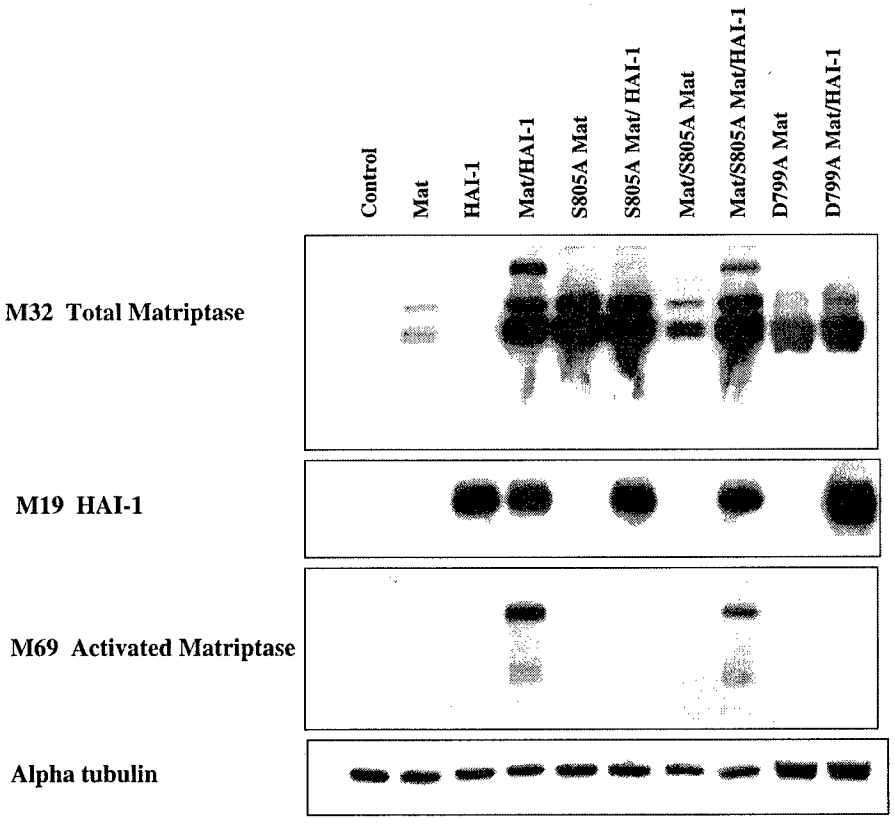

Fig. 6. Cotransfection of HAI-1 with matriptase or transfection of matriptase mutants altered in active site triads or in substrate binding pocket, enhanced the protein expression of matriptase in BT549 cells. BT549 breast cancer cells were transfected with control pcDNA3.1 vector (Control), wild-type matriptase cDNA (Mat), HAI-1 cDNA (HAI-1), both wild-type matriptase and HAI-1 cDNAs simultaneously (Mat/HAI-1), matriptase inactive triad mutant cDNA alone (S805A Mat), both matriptase inactive triad mutant and HAI-1 cDNAs simultaneously (S805A Mat/HAI-1), both wild-type matriptase and matriptase inactive triad mutant cDNAs simultaneously (Mat/S805A Mat), combination of wild-type matriptase, matriptase-inactive triad mutant, and HAI-1 cDNAs simultaneously (Mat/S805A Mat/HAI-1), matriptase substrate binding pocket mutant cDNA alone (D799A Mat), or both matriptase substrate binding pocket mutant and HAI-1 cDNAs simultaneously (D799A Mat/HAI-1), as indicated. Total matriptase was detected by Western blot analysis with the M32 MAb. HAI-1 was detected by immunoblot analysis with the use of MAb M19. Activated matriptase was detected with the use of the M69 MAb. Activated protease could also be detected by formation of the 120-kDa matriptase: HAI-1 complex. Protein loading was monitored by using an α-tubulin-specific MAb.

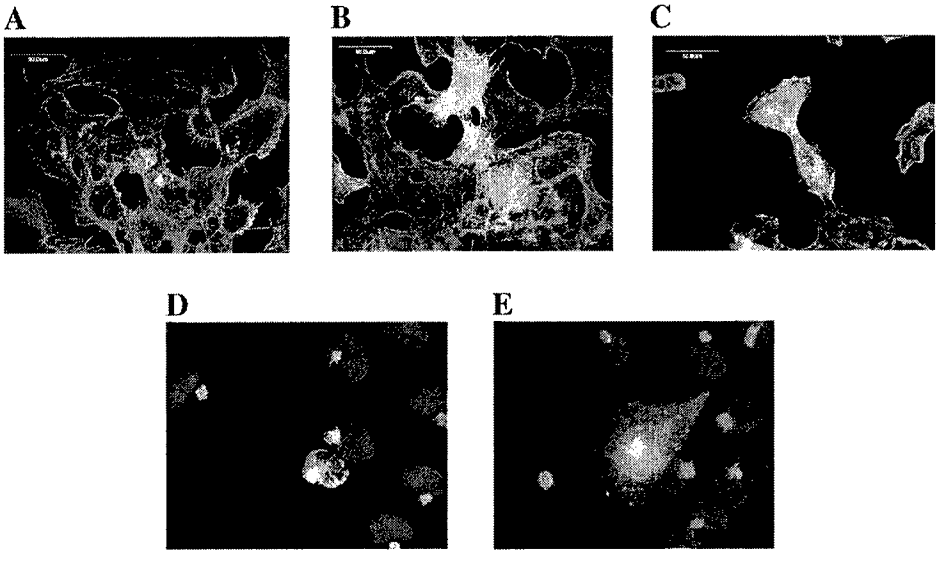

Fig. 7. The poor expression of wild-type matriptase in BT549 cells is due to a defect in matriptase intracellular trafficking. BT549 breast cancer cells were transfected with matriptase alone (*A*), matriptase plus HAI-1 (*B*), or with the catalytically inactive triad mutant S805A matriptase (*C*) and stained for matriptase using the M32 MAb and FITC-labeled rabbit anti-mouse IgG (green) and for actin using Texas red-conjugated phalloidin (red). To demonstrate more clearly the site of the matriptase trafficking defect, matriptase was transfected alone (*D*) or with HAI-1 (*E*), and cells were stained for matriptase using Alexa Fluor 594-conjugated M32 MAb (red), for the Golgi-specific protein GM130 by using a GM130-specific MAb and FITC-conjugated rabbit anti-mouse IgG (green), and for nuclei using 4,6-diamidino-2-phenylindole (blue). Data are representative of 3 independent experiments.

matriptase) were achieved in BT549 breast cancer cells independently of the presence of HAI-1 (Fig. 6). Whereas high levels of matriptase expression were achieved for the matriptase mutant altered in its active site triad, coexpression of the inactive catalytic triad matriptase mutant with wild-type matriptase caused poor expression of both matriptase species (Fig. 6). These results suggest that unopposed matriptase proteolytic activity could be toxic for its expression, and that the presence of HAI-1 corrects this toxic effect, leading to higher levels of matriptase expression.

Immunofluoresence staining using Texas red conjugated phalloidin (actin), together with the anti-matriptase MAb M32 (Fig. 7*A*), suggested that the poor expression was due to a defect in intracellular trafficking of the protease. Indeed, immunofluorescence using an antibody that recognizes GM130, a Gogi-specific marker, together with Alexa Fluor 594-conjugated M32 matriptase-specific antibody (Fig. 7*D*), revealed that matriptase accumulated in a cellular location consistent with the endoplasmic reticulum and Golgi apparatus. This defect in trafficking was corrected by cotransfection with HAI-1, as shown by immunofluorescence (Fig. 7, *B* and *E*), and resulted in much improved expression of the protease, as shown by Western blot analysis (Fig. 7*A*). In addition, catalytically inactive mutants of matriptase altered in the catalytic triad (S805A matriptase) were able to traffic in the absence of HAI-1 (Fig. 7*C*).

The proper trafficking and enhanced expression of the matriptase protein when cotransfected with HAI-1 was not a phenomena limited to BT549 cells. Indeed, it was also seen when matriptase and HAI-1 were transfected into MDA MB-231 and MDA MB-435 cancer cells (data not shown). These two cancer cell lines also neither express matriptase nor HAI-1 (22).

The first Kunitz domain, but not the second, is required for HAI-1 to facilitate matriptase intracellular trafficking. Because the intracellular trafficking of matriptase depended on HAI-1, we were able to test which domains in HAI-1 were essential for protease expression and trafficking. HAI-1 contains two Kunitz-type serine protease inhibitory domains, Kunitz domain I (at the amino terminus) and Kunitz domain II (at the carboxyl terminus), with an intervening LDL receptor class A domain (25) (Fig. 7).

The Kunitz domain is an ~60 amino acid long-serine protease inhibitory domain for which the bovine basic pancreatic trypsin inhibitor represents the prototypic structure (4). The P1 residue of Kunitz-type inhibitory domains (the amino acid residue COOH terminal to the second conserved cysteine residue) is recognized as the active center responsible for the inhibitory specificity. For example, the corresponding amino acid residues in the Kunitz domains of HAI-1 are Arg260 in domain I and Lys385 in domain II, and therefore these Kunitz domains are predicted to be specific for trypsin-like serine proteases, such as matriptase (interacting with Asp799 in the substrate binding pocket of matriptase). Mutation of Arg260 will completely abolish the inhibitory activity of a Kunitz domain. A previous study (6) of molecular modeling strongly suggested that the first, but not the second, Kunitz domain of HAI-1 is responsible for the inhibition of matriptase. In addition to Arg260, Arg258 was also suggested to be crucial for the activity of Kunitz domain I. Therefore, we constructed point mutations at these three critical basic residues in Kunitz domains I and II (Arg258, Arg260, and Lys385).

Mutation of critical arginine residues in the first Kunitz domain of HAI-1 (R258L and R260L HAI-1) completely abolished the ability of HAI-1 to facilitate the intracellular trafficking of matriptase (Fig. 8). In cotransfection experiments with these HAI-1 mutants, matriptase was poorly expressed, as determined by M32 Western blot analysis, and did not traffic properly, as determined by matriptase immunofluorescence (data not shown). However, mutation of a critical basic residue in the second Kunitz domain of HAI-1 (K385L HAI-1) did not affect the ability of HAI-1 to facilitate matriptase trafficking because matriptase expressed well (Fig. 8) and underwent proper intracellular trafficking, as confirmed by matriptase immunofluorescence (data not shown). It should be noted that the R258L and R260L HAI-1 mutants did not express well when transfected with matriptase compared with the K385L HAI-1 mutant

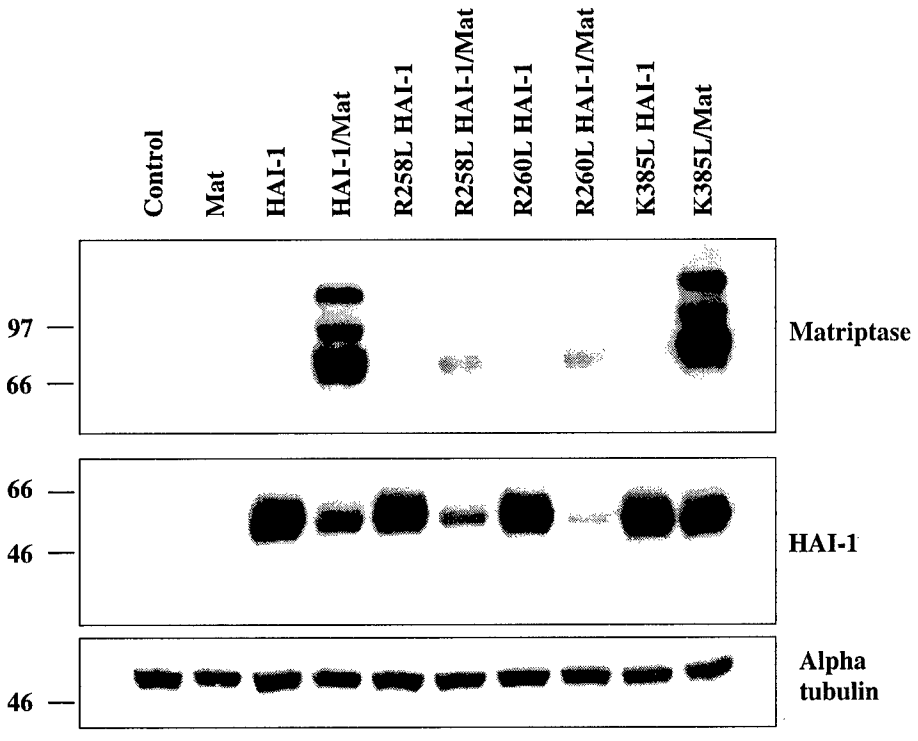

Fig. 8. The facilitation of matriptase trafficking by HAI-1 requires HAI-1 Kunitz domain I, but not Kunitz domain II. BT549 breast cancer cells were transfected with control pcDNA3.1 vector (Control), matriptase cDNA (Mat), HAI-1 cDNA (HAI-1), both matriptase and HAI-1 cDNAs simultaneously (Mat/HAI-1) or with HAI-1 mutants (R258L HAI-1, R260L HAI-1, and K385L HAI-1) transfected with or without wild-type matriptase, as indicated. Matriptase was detected by Western blot analysis with the M32 MAb. HAI-1 transfection was demonstrated by Western blot analysis using the M19 MAb, and equal protein loading was confirmed by using an α-tubulin-specific MAb.

(Fig. 8). However, this was caused by the defect in intracellular trafficking created by unopposed matriptase activity, and not by poor transfection efficiency, as determined by immunofluorescence staining (data not shown). These results suggest that matriptase proteolytic activity, which could result from spontaneous activation in the absence of HAI-1, generally interferes with the trafficking of transmembrane proteins when transfected in the absence of HAI-1.

DISCUSSION

On the basis of our current and previous studies, we have demonstrated that the role of HAI-1 in the regulation of matriptase activity begins at the biosynthesis of the protease, acts throughout the activation and inhibition of the enzyme, and culminates in the final shedding of the protease. This close relationship of the cognate pair, from the cradle to the grave, could be a result of the potent proteolytic activity and the autoproteolytic activation of the protease. The use of HAI-1 as an endogenous inhibitor provides a means to prevent unwanted proteolysis and the subsequent harmful effects of matriptase to cells. The autoproteolytic activation of matriptase appears to require the presence of HAI-1 with matriptase at all times, even at the stage of biosynthesis. In the absence of HAI-1, the biosynthesis of matriptase can only reach a low level, due to an autoproteolytic activation that appears to occur in the Gogi-endoplasmic reticulum apparatus, leading to a harmful effect on the trafficking of the protease, and the cessation of further matriptase translation. In the presence of HAI-1, however, the uncontrolled spontaneous autoproteolytic activation does not occur, or is instead immediately quenched to the levels that allow proper trafficking of matriptase.

Reduced expression of HAI-1 by siRNA resulted in spontaneous activation of matriptase. This suggests that maintenance of a high HAI-1-to-matriptase ratio is necessary to prevent uncontrolled spontaneous matriptase activation. We have estimated that 184 A1N4 mammary epithelial cells express 10 times as much HAI-1 as matriptase (13). During S1P-induced matriptase activation, decreased levels of bioavailable HAI-1, achieved either by siRNA technology or by interference of HAI-1 function by anti-HAI-1 MAb M19, could result in a less efficient inhibition of active matriptase. This could result in the enhanced activation of latent matriptase by active matriptase, due to the perfect match between its cleavage site preference and the flanking sequences of the activation motif of matriptase. Thus there may be two different mechanisms at work to activate matriptase: latent matriptase activating latent matriptase and active matriptase activating latent matriptase. The former occurs to create the initial active matriptase molecules, and only occurs when the active site triad of matriptase is intact (24). The latter could serve to extend the activation level, and is regulated by the level or accessibility of HAI-1.

It seems paradoxical that matriptase activation requires its cognate inhibitor, HAI-1. This function of HAI-1 in matriptase activation requires its LDL receptor class A domain (24). In contrast, the Kunitz domain I of HAI-1 is essential for matriptase expression and inhibition (12). The participation of HAI-1 in matriptase activation allows HAI-1 to have direct access to active matriptase. This would ensure that HAI-1 is present at the site of matriptase activation, ready to inhibit the active protease. This close connection between matriptase activation and inhibition could allow the active protease only a very short period of time to act on its substrates, thus maintaining tight control of the enzyme. It is plausible that

matriptase substrates may also be involved in matriptase activation, in a similar fashion to the inhibitor. This would allow matriptase to activate its substrates before HAI-1 inhibited the protease.

In our previous studies (1), activation of matriptase was followed by the shedding of matriptase and HAI-1 from the cell surface. The membrane-bound 120-kDa matriptase-HAI-1 complex contains the full-length HAI-1 and the 70-kDa matriptase. Cleavage at HAI-1 to release its ectodomains from the transmembrane domain is required for shedding of the matriptase-HAI-1 complex. In light of the connection between matriptase activation and shedding of matriptase-HAI-1 complex (1), the protease(s) responsible for the shedding may be one (or more) of the substrates of matriptase.

In conclusion, HAI-1 is not only the cognate inhibitor of matriptase, but also is involved in almost every aspect of matriptase functionality and regulation. We now show that HAI-1 is essential for preventing spontaneous activation of matriptase. This function is important for the intracellular trafficking and proper expression of the protease. This also prevents uncontrolled activation of the protease. HAI-1 seems to simultaneously participate in autoproteolytic activation and inhibition of matriptase, and has a role in subsequent shedding of the protease. The regulation of matriptase by HAI-1 from its biosynthesis to its removal from the cell surface may ensure that this potentially hazardous enzyme functions properly, thus avoiding harmful effects.

GRANTS

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